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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/807,877	04/19/2001	David Ian Stapleton	4050,000900	7462

7590 09/29/2003

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EXAMINER	
YANG, NELSON C	
ART UNIT	**PAPER NUMBER**
1641	

DATE MAILED: 09/29/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Applicati n No.	Applicant(s)	
	09/807,877	STAPLETON ET AL.	
	Examiner	Art Unit	
	Nelson Yang	1641	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondenc address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on 09 June 2003 .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 1-24 is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) 1-24 are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

Election/Restrictions

1. Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

2. In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

Group I, claim(s) 1, 2, 5, 8, 9, 10, 22, 23, drawn to a method of identifying modulators of AMPK-mediated activation of a nitric oxide synthase enzyme comprising the special technical feature of testing putative modulators for their ability to mediate the phosphorylation of Ser-1177 depending on calmodulin and calcium ion concentrations.

Group II, claim(s) 3, 4, 6, 7, 9, 11, 22, 24, drawn to a method of identifying modulators of AMPK-mediated inhibition of a nitric oxide synthase enzyme having the special technical feature of testing putative modulators for their ability to mediate the phosphorylation of Thr-495 in the presence of limiting calcium ions.

Group III, claim(s) 1, 5, 6, 7, 12, 13, drawn to drawn to a method of identifying modulators of AMPK-mediated activation of a nitric oxide synthase enzyme having the special technical feature of testing putative modulators for their ability to mediate the phosphorylation of Ser-1417.

Group IV, claim(s) 14, 15, 17, 19-21, drawn to an antibody having the special technical feature of phosphorylating eNOS at Ser-1177.

Group V, claim(s) 14, 16, 18, 19-21 drawn to an antibody having the special technical feature of phosphorylating eNOS at Thr-495.

3. The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special

technical features for the following reasons: The application contains claims to more than one of the combinations of categories of inventions as set forth by 37 CFR 1.475.

According to 37 CFR 1.475 regarding unity of invention:
(a) An international and a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in an application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

(b) An international or a national stage application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to one of the following combinations of categories:
(1) A product and a process specially adapted for the manufacture of said product; or
(2) A product and a process of use of said product; or
(3) A product, a process specially adapted for the manufacture of the said product, and a use of the said product; or
(4) A process and an apparatus or means specifically designed for carrying out the said process; or
(5) A product, a process specially adapted for the manufacture of the said product, and an apparatus or means specifically designed for carrying out the said process.

If an application contains claims to more or less than one of the combinations of categories of invention set forth in paragraph (b) above, unity of invention might not be present. Furthermore, the determination whether a group of inventions is so linked as to form a single general inventive concept shall be made without regard to whether the inventions are claimed in separate claims or as alternatives within a single claim.

4. Unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more special technical features. The term "special technical features" is defined as meaning those technical features that define a contribution which each of the inventions considered as a whole, makes over the prior art. The determination is made based on the contents of the claims as interpreted in light of the description and drawings. In the instant application, Groups I-V have differing special technical features:

Group I has the special technical feature of testing putative modulators for their ability to mediate the phosphorylation of Ser-1177 depending on calmodulin and calcium ion concentrations.

Group II has the special technical feature of testing putative modulators for their ability to mediate the phosphorylation of Thr-495 in the presence of limiting calcium ions.

Group III has the special technical feature of testing putative modulators for their ability to mediate the phosphorylation of Ser-1417 depending on calmodulin and calcium ion concentrations.

Group IV has the special technical feature of an antibody phosphorylating eNOS at Ser-1177.

Group V has the special technical feature of an antibody phosphorylating eNOS at Thr-495.

5. Furthermore, the methods of group I and II are anticipated by Ju et al [H Ju, R Zou, VJ Venema, RC Venema, *Direct Interaction of Endothelial Nitric-oxide Synthase and Caveolin-1 Inhibits Synthase Activity,* Communication, 1997, 272(30):18522-18525].

6. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Sequence Requirements

7. This application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 C.F.R. § 1.821(a)(1) and (a)(2).

However, this application fails to comply with the requirements of 37 C.F.R. §§ 1.821-1.825 for the reason(s) set forth on the Raw Sequence Listing Error Report.

Any questions regarding compliance with the sequence rules requirements specifically should be directed to the departments listed at the bottom of the Notice to Comply.

8. **APPLICANT IS GIVEN THE TIME ALLOTED IN THIS LETTER WITHIN WHICH TO COMPLY WITH THE SEQUENCE RULES, 37 C.R.F. §§ 1.821-1.825.** Failure to comply with these requirements will result in ABANDONMENT of the application under 37 C.F.R. § 1.821(g). Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 C.F.R. § 1.136. In no case may an applicant extend the period for response beyond the six month statutory period. Direct the response to the undersigned. Applicant is requested to return a copy of the attached Notice to Comply with the response.

9. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Nelson Yang whose telephone number is 703-305-4508. The examiner can normally be reached on 8:30-5:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Long V Le can be reached on 703-305-3399. The fax phone number for the organization where this application or proceeding is assigned is (703) 872-9306.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0196.

NY

Long Le

LONG V. LE
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

07/28/03

Notice to Comply	Application No. 09/807877	Applicant(s) Stapleton, Chen, Michell, Kemp, Mitchelhill	
	Examiner Nelson Yang	Art Unit 1641	Paper No 6

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Applicant must file the items indicated below within the time period set the Office action to which the Notice is attached to avoid abandonment under 35 U.S.C. § 133 (extensions of time may be obtained under the provisions of 37 CFR 1.136(a)).

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

☒ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998).

☒ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).

☒ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).

☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."

☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).

☐ 7. Other: _

Applicant Must Provide:

☒ An initial or **substitute** computer readable form (CRF) copy of the "Sequence Listing".

☒ An initial or **substitute** paper copy of the "Sequence Listing", as well as an amendment directing its entry into the **specification**.

☒ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:

For Rules Interpretation, call (703) 308-4216 or (703) 308-2923
For CRF Submission Help, call (703) 308-4212
Patent Software Program Support
Technical Assistance.......................................703-287-0200
To Purchase Patent Software.........................703-306-2600

PLEASE RETURN A COPY OF THIS NOTICE WITH YOUR REPLY

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Communication

Direct Interaction of Endothelial Nitric-oxide Synthase and Caveolin-1 Inhibits Synthase Activity*

(Received for publication, March 31, 1997, and in revised form, May 23, 1997)

Hong Ju, Rong Zou, Virginia J. Venema, and Richard C. Venema‡

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Endothelial nitric-oxide synthase (eNOS) and caveolin-1 are associated within endothelial plasmalemmal caveolae. It is not known, however, whether eNOS and caveolin-1 interact directly or indirectly or whether the interaction affects eNOS activity. To answer these questions, we have cloned the bovine caveolin-1 cDNA and have investigated the eNOS-caveolin-1 interaction in an *in vitro* binding assay system using glutathione *S*-transferase (GST)-caveolin-1 fusion proteins and baculovirus-expressed bovine eNOS. We have also mapped the domains involved in the interaction using an *in vivo* yeast two-hybrid system. Results obtained using both *in vitro* and *in vivo* protein interaction assays show that both N- and C-terminal cytosolic domains of caveolin-1 interact directly with the eNOS oxygenase domain. Interaction of eNOS with GST-caveolin-1 fusion proteins significantly inhibits enzyme catalytic activity. A synthetic peptide corresponding to caveolin-1 residues 82–101 also potently and reversibly inhibits eNOS activity by interfering with the interaction of the enzyme with Ca^{2+}/calmodulin (CaM). Regulation of eNOS in endothelial cells, therefore, may involve not only positive allosteric regulation by Ca^{2+}/CaM, but also negative allosteric regulation by caveolin-1.

Plasmalemmal caveolae are small membrane invaginations present in most cells of higher eukaryotes. These membrane specializations appear to function both as endocytotic carriers and as signal transduction organizing centers. In the latter case, caveolae compartmentalize a subset of signal-transducing molecules in membrane microdomains at the cell surface (1, 2). A major structural protein of caveolae is caveolin, a 21–24-kDa integral membrane protein that occurs in three homologous, but distinct isoforms termed caveolins-1, -2, and -3 (3). Full-length caveolin-1 contains three domains: a 101-residue N-terminal domain, a 33-residue membrane-spanning region, and a 44-residue C-terminal domain. The N- and C-terminal domains of caveolin-1 face the cytoplasm suggesting that the membrane-spanning region forms a hairpin loop within the membrane (4–6). A cytosolic membrane-proximal subdomain of the N-terminal domain (residues 82–101) interacts directly with Gα subunits, Ha-Ras, and Src family tyrosine kinases (7–9). Interaction of these signaling proteins with this caveolin-1 scaffolding domain serves to sequester the proteins in caveolae and to inhibit or suppress their catalytic activities.

Another important signaling protein known to be localized in caveolae is endothelial nitric-oxide synthase (eNOS)[1] (10, 11). Production of NO by eNOS in endothelial caveolae appears to play a key role in modulating vascular tone, platelet aggregation, leukocyte adhesion, vascular smooth muscle cell proliferation, and vascular lesion formation (12, 13). eNOS has a bidomain structure consisting of an N-terminal oxygenase domain and a C-terminal reductase domain (14). Located between the oxygenase and reductase domains is a Ca^{2+}/calmodulin (CaM)-binding region (15). Association of eNOS and caveolin-1 in cultured bovine endothelial cells has been demonstrated previously in coimmunoprecipitation experiments (16, 17). It is not known, however, whether eNOS and caveolin-1 interact directly or indirectly (*i.e.* through an adaptor protein). Also unidentified are the interacting domains, if any, in the two proteins. Most importantly, the functional consequences of caveolin-1 binding on eNOS catalytic activity have not been determined. Each of these questions with regard to the eNOS-caveolin-1 protein-protein interaction has been addressed in the present study.

EXPERIMENTAL PROCEDURES

Materials—The glutathione *S*-transferase (GST)-fusion protein cloning vector, pGEX-4T-1, CaM-Sepharose 4B, and anti-GST polyclonal antibody were obtained from Pharmacia Biotech Inc. Sf9 insect cells were purchased from Pharmingen (San Diego, CA) and maintained in serum-supplemented Hink's TNM-FH media from Mediatech, Inc. (Herndon, VA). Monoclonal antibody to eNOS (clone 3) was purchased from Transduction Laboratories (Lexington, KY). L-[^{14}C]Arginine and ECL reagents came from Amersham Corp. pGBT9 (DNA binding domain hybrid cloning vector), pGAD424 (activation domain hybrid cloning vector), and *Saccharomyces cerevisiae* SFY526 were obtained from CLONTECH (Palo Alto, CA). Oligonucleotide primers for PCR, 5'-RACE kit, and TriZOL reagent were purchased from Life Technologies Inc. TA Cloning kit was obtained from Invitrogen (Carlsbad, CA). Protein assay kit was purchased from Bio-Rad. Bovine CaM came from Sigma. Synthetic peptides were obtained from Research Genetics, Inc. (Huntsville, AL) and were >95% pure as determined by high performance liquid chromatography.

Cloning of the cDNA Encoding Bovine Caveolin-1—Total RNA from cultured bovine aortic endothelial cells was isolated with TriZOL reagent and subjected to reverse transcription-polymerase chain reaction. The upstream primer for PCR was based on the first 20 nucleotides of the caveolin-1 coding sequence that are identical in sequences previously cloned from dog, mouse, and human (18–20). The downstream primer used was an oligo(dT)$_{17}$ oligonucleotide. PCR amplification produced a 568-base pair fragment that was subcloned into the TA cloning vector and sequenced in the Molecular Biology Core Facility of the Medical College of Georgia. Three independent PCR reactions produced identical nucleotide sequences ruling out the possibility of PCR-associated nucleotide incorporation errors. To confirm that the first 20 nucleotides of the bovine coding sequence are identical to the sequence of the

* This work was supported by National Institutes of Health Grant HL57201 (to R. C. V.) and by grants-in-aid from the Georgia Affiliate of the American Heart Association (to R. C. V.) and the American Heart Association National Center (to R. C. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: eNOS, endothelial nitric-oxide synthase; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; RACE, rapid amplification of cDNA ends; CaM, calmodulin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

upstream primer, 5′-RACE (rapid amplification of cDNA ends) was performed with a 5′-RACE kit (Life Technologies Inc.). The sequence obtained by 5′-RACE was identical to that obtained in the initial PCR. The nucleotide sequence has been submitted to the GenBank™/EMBL Data Bank with accession number U86639.

Construction and Purification of GST-Caveolin-1 Fusion Proteins—cDNA constructs encoding GST-caveolin-1 fusion proteins were created by subcloning into the GST-fusion protein cloning vector, pGEX-4T-1. Caveolin-1 cDNA sequences encoding full-length caveolin-1 (residues 1–178) and caveolin-1 residues 1–60, 1–101, 102–134, and 135–178 were generated by PCR amplification of the full-length bovine sequence cloned into the TA cloning vector. Primers for PCR were designed to incorporate 5′ *Eco*RI and *Sal*I restriction sites for subcloning. The cDNAs encoding the fusion proteins were sequenced to confirm the creation of in-frame fusions devoid of PCR-associated nucleotide incorporation errors. Fusion proteins and a GST-nonfusion protein were expressed in *Escherichia coli* and purified by affinity chromatography on glutathione-agarose as described by Frangioni and Neel (21).

Expression and Purification of eNOS in a Baculovirus System—Bovine eNOS was expressed in a baculovirus/Sf9 insect cell system and purified to >95% homogeneity as described previously (15, 22). eNOS was purified in buffers containing 2 mM EGTA, and purified enzyme was completely dependent on exogenous CaM for activity.

Interaction of Recombinant Baculovirus-expressed eNOS with GST-Caveolin-1 Fusion Proteins—GST or GST-caveolin-1 fusion proteins (100 pmol each, quantitated by Bio-Rad protein assay) prebound to glutathione-agarose beads were washed three times in buffer containing 50 mM Tris-HCl, pH 7.4, 20% glycerol, and the following protease inhibitors: 1% phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 5 μg/ml aprotinin. Equimolar amounts of fusion proteins in each condition were confirmed by immunoblotting with anti-GST antibody. Washed beads were incubated overnight (with shaking at 4 °C) in 1 ml of the above buffer containing 100 pmol of bovine eNOS, expressed and purified from a baculovirus system as described previously (22). Following the overnight binding reaction, beads were washed six times in 1 ml of 50 mM Hepes, pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.5% CHAPS plus the protease inhibitors listed above. Bound proteins were eluted with 100 μl of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 100 mM reduced glutathione, plus protease inhibitors. Eluted proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-eNOS monoclonal antibody as described previously (23). Binding experiments were also performed with GST or GST-caveolin-1 fusion proteins and purified bovine CaM. In one set of experiments the binding assay was carried out exactly as described above for eNOS. In another set of experiments, EDTA was omitted from all buffers and replaced with 2 mM $CaCl_2$.

Interaction of eNOS and Caveolin-1 Domains in a Yeast Two-hybrid System—Construction of bovine eNOS oxygenase domain (residues 1–505) and reductase domain (residues 506–1205) hybrids with the GAL4 DNA binding and activation domains has been described previously (24). Bovine caveolin-1 hybrids encoding residues 1–178, 1–101, and 135–178 were created by subcloning into the shuttle/expression vectors, pGBT9 and pGAD424 (CLONTECH). DNA sequences for subcloning were generated by PCR amplification of the full-length bovine caveolin-1 cDNA in the TA cloning vector. PCR primers were designed to incorporate 5′ *Eco*RI and *Sal*I restriction sites into the amplified products to facilitate subcloning. All DNA constructs were sequenced to confirm the creation of an in-frame hybrid devoid of PCR-associated nucleotide incorporation errors. Transformation of yeast and colony lift filter assay of β-galactosidase activity has been described previously (24). All DNA-binding domain and activation domain fusion constructs were confirmed not to reconstitute GAL4 activity by themselves, and all activation domain fusion constructs were confirmed not to activate transcription when combined with the unrelated pLAM 5′ (human lamin C_{66-230} in pGBT9).

Determination of the Effects of GST-Caveolin-1 Fusion Proteins and Synthetic Peptides on eNOS Activity—GST or GST-caveolin-1 fusion proteins were eluted from glutathione-agarose beads with reduced glutathione, and 300 pmol of each protein (quantitated by Bio-Rad protein assay) was incubated for 5 min at 37 °C with purified, baculovirus-expressed bovine eNOS (100 pmol) in 50 mM Tris-HCl, pH 7.5, buffer. eNOS activity was then determined by monitoring the rate of formation of L-[^{14}C]citrulline from L-[^{14}C]arginine (50 μM) in the presence of excess cofactors including Ca^{2+} (2 mM), purified bovine CaM (250 pmol), NADPH (1 mM), FAD (4 μM), FMN (4 μM), and tetrahydrobiopterin (40 μM) in a 200-μl reaction volume as described previously (22). Experiments testing the effects of synthetic peptides were carried out using the same protocol and various concentrations of peptides.

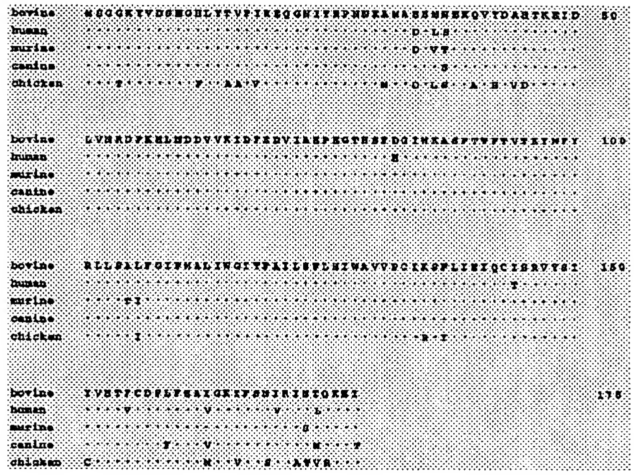

FIG. 1. **Comparison of the caveolin-1 amino acid sequences from bovine, human, murine, canine, and chicken.** Data base accession numbers are bovine (U86639), human (Z18951), murine (U07645), canine (Z12161), and chicken (A46424).

CaM-Sepharose Chromatography—Purified eNOS (1 μg) was incubated with or without synthetic peptides (10 μM) in 50 mM Tris-HCl, pH 7.5, buffer for 5 min at 37 °C and then subjected to CaM-Sepharose chromatography as described previously (22). The amount of eNOS eluted from the CaM-Sepharose column was quantitated by immunoblotting with anti-eNOS antibody as described previously (23).

RESULTS AND DISCUSSION

eNOS and caveolin-1 are known to be associated in cultured bovine aortic and lung microvascular endothelial cells (16, 17). It is not known, however, whether they interact directly or indirectly (*i.e.* through an adaptor protein). To answer this question for purified, baculovirus-expressed bovine eNOS, we have isolated and sequenced the cDNA encoding bovine caveolin-1. Analysis of the deduced amino acid sequence indicates that bovine caveolin-1 shares 94, 96, 97, and 86% identity with the human, murine, canine, and chicken sequences, respectively (18–20, 25) (Fig. 1). To determine whether eNOS interacts directly with caveolin-1, we expressed full-length bovine caveolin-1 as a GST-fusion protein in *E. coli*. In addition, to determine which domains of caveolin-1 are involved in eNOS binding, we also expressed GST-fusion proteins of caveolin-1 residues 1–60, 1–101 (N-terminal cytoplasmic domain), 102–134 (membrane-spanning domain), and 135–178 (C-terminal cytoplasmic domain). The fusion proteins and a GST-nonfusion protein were purified by affinity chromatography on glutathione-agarose. The GST-caveolin-1 fusions or GST alone prebound to agarose beads were then used in *in vitro* binding assays with recombinant bovine eNOS, expressed and purified from a baculovirus system (22). Beads were incubated with eNOS at 4 °C overnight and extensively washed, and bound proteins were eluted with reduced glutathione. Eluted proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-eNOS antibody. As shown in Fig. 2, eNOS bound specifically to the full-length GST-caveolin-1 fusion protein but not to GST alone, demonstrating that eNOS and caveolin-1 interact directly. Furthermore, eNOS bound specifically to GST-fusions containing only the N-terminal caveolin-1 cytoplasmic domain (residues 1–101) or only the C-terminal caveolin-1 cytoplasmic domain (residues 135–178). In contrast, GST-fusions containing only caveolin-1 residues 1–60 or the caveolin-1 membrane-spanning domain (residues 102–134) did not bind to eNOS. The eNOS-caveolin-1 association thus appears to involve binding of eNOS to both

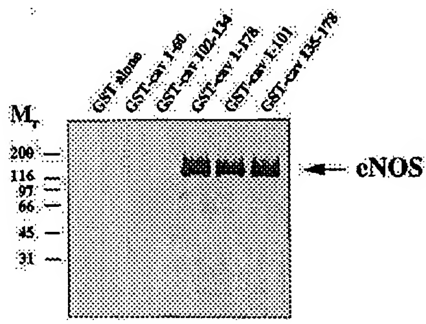

FIG. 2. ***In vitro* binding of eNOS to GST-caveolin-1 fusion proteins.** GST-caveolin-1 fusion proteins representing full-length caveolin-1 (*GST-cav 1-178*), the caveolin-1 N-terminal cytoplasmic domain (*GST-cav 1-101*), caveolin-1 residues 1–60 (*GST-cav 1-60*), the caveolin-1 C-terminal cytoplasmic domain (*GST-cav 135-178*), the caveolin-1 membrane-spanning domain (*GST-cav 102-134*), plus GST alone were expressed in *E. coli* and purified by affinity binding to glutathione-agarose beads. Proteins prebound to beads were incubated with purified baculovirus-expressed recombinant eNOS. Following binding, extensive washing, and elution with reduced glutathione, proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-eNOS antibody. The result shown is a representative immunoblot from three separate experiments.

cytoplasmic tails of caveolin-1. Furthermore, either cytoplasmic domain of caveolin-1 by itself is sufficient to mediate the eNOS binding.

To verify the conclusions reached based on the *in vitro* binding assays and to determine whether caveolin-1 binds to either the eNOS oxygenase domain or the eNOS reductase domain, or both, we have also investigated the eNOS-caveolin-1 interaction in a yeast two-hybrid system. Hybrid cDNA constructs were prepared that encoded full-length bovine caveolin-1 (residues 1–178), the caveolin-1 N-terminal cytoplasmic domain (residues 1–101), the caveolin-1 C-terminal cytoplasmic domain (135–178), the bovine eNOS oxygenase domain (1–505), and the bovine eNOS reductase domain (506–1205) fused to either the GAL4 DNA binding domain or activation domain. Various pairwise combinations of the plasmid constructs were used to cotransform the yeast strain, SFY526. Interactions of hybrid proteins were assessed by colony lift filter assay of β-galactosidase reporter gene transcription. As shown in Table I, caveolin-1 interacted with itself in the two-hybrid system through both N- and C-terminal cytoplasmic domains, as has been demonstrated previously in *in vitro* binding assays with GST-fusion proteins (26, 27). Furthermore, both N- and C-terminal domains of caveolin-1 interacted with eNOS in the two-hybrid system, confirming the results obtained in the GST-fusion protein binding assays. Caveolin-1 interactions were restricted to the eNOS oxygenase domain and did not occur with the eNOS reductase domain.

To determine whether interaction of eNOS with caveolin-1 alters nitric-oxide synthase activity, we incubated equal quantities of purified, baculovirus-expressed eNOS with equimolar quantities of the GST alone, GST-caveolin 1–60, GST-caveolin 102–134, GST-caveolin 1–178, GST-caveolin 1–101, and GST-caveolin 135–178 fusion proteins. eNOS activity was then determined by arginine-to-citrulline conversion assay in the presence of excess cofactors, Ca^{2+}, and CaM. As shown in Fig. 3, the full-length caveolin-1 fusion protein inhibited eNOS activity by about 60%. Furthermore, either of the caveolin-1 cytoplasmic domains appears to be sufficient to mediate eNOS inhibition because the GST-caveolin 1–101 and GST-caveolin 135–178 fusion proteins also inhibited enzyme activity by about 60%. In contrast, the GST-caveolin 1–60 and GST-caveolin 102–134 fusion proteins were without effect on activity. To confirm that

TABLE I
Interactions between eNOS and caveolin-1 in a yeast two-hybrid system

Pairwise combinations of hybrid plasmids were used to cotransform yeast cells. Cotransformants were assayed for β-galactosidase activity by the colony lift filter assay method using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as substrate. Similar results were obtained in five separate transformations.

Binding domain hybrid	Activation domain hybrid	β-Galactosidase activity
Caveolin-(1–178)	Caveolin-(1–178)	+
Caveolin-(1–178)	Caveolin-(1–101)	+
Caveolin-(1–178)	Caveolin-(135–178)	+
eNOS-(1–505)	Caveolin-(1–178)	+
eNOS-(1–505)	Caveolin-(1–101)	+
eNOS-(1–505)	Caveolin-(135–178)	+
eNOS-(506–1205)	Caveolin-(1–178)	−
eNOS-(506–1205)	Caveolin-(1–101)	−
eNOS-(506–1205)	Caveolin-(135–178)	−

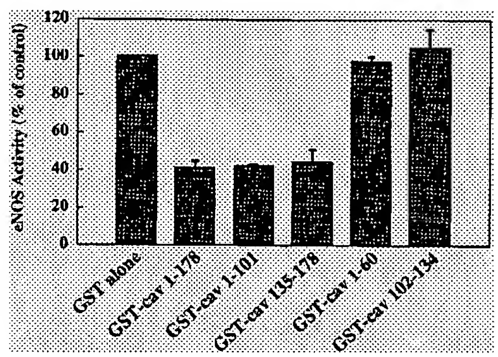

FIG. 3. **Effect of GST-caveolin-1 fusion proteins on eNOS catalytic activity.** GST or GST-caveolin-1 fusion proteins were eluted from glutathione-agarose beads and incubated with purified baculovirus-expressed eNOS for 5 min at 37 °C. eNOS activity was then determined by arginine-to-citrulline conversion assay. Results shown are the mean ± S.E. of triplicate determinations from three different experiments.

inhibition was due to binding of the fusion proteins to eNOS rather than to CaM, we also performed *in vitro* binding assays with CaM and GST-caveolin-1 fusion proteins under the same conditions used in the eNOS binding studies. In addition, we performed *in vitro* binding assays of eNOS and CaM in which 1 mM EDTA was omitted from all buffers and replaced by 2 mM $CaCl_2$. In both sets of experiments, no CaM binding to any of the GST-caveolin-1 fusion proteins was detected.

Inhibition by GST-caveolin 1–101 but not by GST-caveolin 1–60 suggests that the inhibitory region of the N-terminal cytoplasmic domain may correspond to the caveolin-1 scaffolding domain (residues 82–101) previously shown to inhibit $G\alpha$ subunits, Ha-Ras, and Src family tyrosine kinases (7–9). To test this hypothesis we prepared synthetic peptides corresponding to caveolin-1 residues 61–81 and 82–101. As shown in Fig. 4, the 82–101 peptide potently inhibited eNOS activity (IC_{50} ~1 μM). Complete inhibition was observed at a 10 μM concentration of peptide. A 10 μM concentration of the 61–81 peptide, on the other hand, actually increased activity by about 30%. To determine whether inhibition was due to an effect of the 82–101 peptide on the eNOS interaction with Ca^{2+}/CaM, we preincubated eNOS with and without the 61–81 and 82–101 peptides (10 μM) and then subjected the enzyme to CaM-Sepharose chromatography. Enzyme was allowed to bind to the column in the presence of 2 mM $CaCl_2$ and was eluted with 2 mM EGTA. The amount of enzyme eluted in each condition was quantitated by immunoblotting with monoclonal anti-eNOS antibody. As shown in Fig. 5*A*, the 82–101 peptide reduced binding of eNOS to CaM-Sepharose by >90% (as determined by

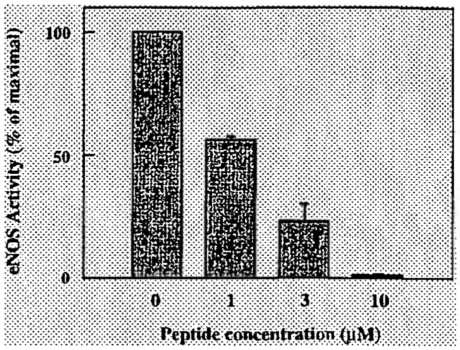

FIG. 4. **Effect of a peptide corresponding to caveolin-1 residues 82–101 on eNOS catalytic activity.** eNOS activity was determined by arginine-to-citrulline conversion assay in the absence and presence of various concentrations of caveolin-1-(82–101) peptide. Results shown are the mean ± S.E. of triplicate determinations from three separate experiments.

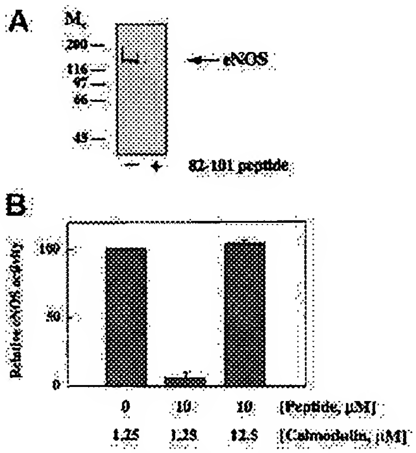

FIG. 5. **Effect of the caveolin-1-(82–101) peptide on eNOS binding to CaM-Sepharose and reversal of eNOS inhibition by excess Ca^{2+}/CaM.** *A*, eNOS was preincubated with and without the caveolin-1-(82–101) peptide and subjected to chromatography on CaM-Sepharose. The enzyme was eluted from CaM-Sepharose with 2 mM EGTA. The amount of eNOS eluted in each condition was quantitated by immunoblotting with anti-eNOS monoclonal antibody. Equivalent results were obtained in three experiments. *B*, eNOS was preincubated for 5 min with and without the caveolin-1-(82–101) peptide (10 μM) and then incubated for an additional 5 min with either 1.25 or 12.5 μM Ca^{2+}/CaM. eNOS activity was then determined by arginine-to-citrulline conversion assay. Results shown are mean ± S.E. of triplicate determinations from three separate experiments.

densitometry of immunoblots). In contrast, the 61–81 peptide had no effect on CaM binding. Furthermore, peptide inhibition of eNOS was reversible by increasing the molar excess of Ca^{2+}/CaM by 10-fold. eNOS was preincubated for 5 min at 37 °C with the 82–101 peptide (10 μM). Enzyme activity was then determined in the presence of the standard molar excess concentration of Ca^{2+}/CaM (1.25 μM) routinely used in the arginine-to-citrulline conversion assay. Activity was confirmed to be completely inhibited in the presence of 10 μM peptide and 1.25 μM Ca^{2+}/CaM. eNOS was then incubated for an additional 5 min at 37 °C with either 1.25 μM or 12.5 μM Ca^{2+}/CaM. eNOS activity was then redetermined. As shown in Fig. 5*B*, increasing the Ca^{2+}/CaM concentration by 10-fold completely reversed the inhibitory effects of the 82–101 peptide.

In summary, the results of the present study provide several important new insights into the eNOS-caveolin-1 interaction. First, eNOS and caveolin-1 interact directly rather than indirectly. Second, interaction involves both the N- and C-terminal cytoplasmic domains of caveolin-1 and is thus fundamentally different from the interaction of caveolin-1 with Gα subunits, Ha-Ras, and Src family tyrosine kinases. Third, the caveolin-1 interaction with eNOS involves only the eNOS oxygenase domain and not the eNOS reductase domain. Fourth, interaction of eNOS with caveolin-1 significantly inhibits eNOS catalytic activity. Finally, inhibition appears to be due to interference with the eNOS interaction with Ca^{2+}/CaM. Regulation of eNOS activity in endothelial cells, therefore, may involve not only positive allosteric regulation by Ca^{2+}/CaM, but also negative allosteric regulation by caveolin-1. It is conceivable that interaction of eNOS with caveolin-1 provides a mechanism to deactivate the enzyme subsequent to its activation by agonist-stimulated elevation of intracellular Ca^{2+}. Protein-protein interactions between eNOS and caveolin-1, however, are probably not sufficient to mediate membrane attachment. Fatty acylation of eNOS by myristate and palmitate appears to also be required. This requirement has been demonstrated in previous studies of an eNOS myristoylation-deficient mutant in which glycine 2 has been mutated to an alanine. The mutant enzyme is neither myristoylated nor palmitoylated and, as a result, is not membrane-associated (22).

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